Science in China Series B: Chemistry

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Effect and mechanism of carbon sources on phosphorus uptake by microorganisms in sequencing batch reactors with the single-stage oxic process

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To investigate the chief reason for phosphorus uptake by microorganisms affected by substrates in sequencing batch reactors with the single-stage oxic process, two typical substrates, glucose (R1) and acetate (R2) were used as the sole carbon source, and the performances of phosphorus removal and the changes of intracellular storage were compared. The experimental results showed that the phenomenon of excess phosphorus uptake was observed in two reactors, but bacteria's capability to take in phosphorus and its intracellular storage were obviously different under the same operational condition. After steady-state operation, total phosphorus (TP) removed per MLVSS in R1 and R2 was 6.7—7.4 and 2.7—3.2 mg/g, respectively. The energy storage of poly-β-hydroxyalkanoates (PHA) was nearly constant in R1 during the whole period, and another aerobic storage of glycogen was accumulated (the max accumulation of glycogen was 3.21 mmol-C/g) when external substrate was consumed, and then was decreased to the initial level. However in R2, PHA and glycogen were both accumulated (2.1 and 0.55 mmol-C/g, respectively) when external substrate was consumed, but they showed different changes after the period of external consumption. Compared to rapid decrease of PHA to the initial level, glycogen continued accumulating to the peak (0.88 mmol-C/g) in 2 h of aeration before decreasing. During the aeration, the accumulations/transformations of internal carbon sources in R1 were higher than those in R2. In addition, obvious TP releases were both observed in R1 and R2 other than PHA and glycogen during the long-term idle period; moreover, the release content of phosphorus in R1 was also higher than that in R2. The researches indicated that different aerobic metabolism of substrate occurred in R1 and R2 due to the different carbon sources in influent, resulting in different types and contents of aerobic storage accumulated/translated in bacteria of R1 and R2. As a result, ATP content provided for phosphorus uptake was different in R1 and R2, and the capability to take up phosphorus was also different from each other.

biological phosphorus removal, poly-β-hydroxyalkanoates, glycogen, sequecing batch reactor, single-stage oxic process

1 Introduction

Phosphate (Pi) is important nutrition for biology, but excessive Pi supply to water will lead to ecosystem balance through a process known as eutrophication. This can lead to deteriorate water quality, increase wastewater treatment costs, and reduce the dissolve oxygen concentration for covering with water surface by algal blooms, thus resulting in a reduction in the biological diversity and the aquiculture. Therefore, efficient and reliable Pi removal methods are very important to the increasingly stringent problem of eutrophication.

Received December 11, 2008; accepted February 27, 2009
doi: 10.1007/s11426-009-0152-6
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Supported by the National Natural Science Foundation of China (Grant No. 50478054) and the Program for NCET in University (Grant No. 0770)

Compared to chemical Pi removal, biological Pi removal has attracted more researchers' attention due to its lower operational cost and lower environmental impact[1]. The essence of biological Pi removal is to exploit the ability of certain microorganisms to accumulate Pi in excess of metabolic requirement and to store this intracellularly as the biopolymer polyphosphate (poly-P) under certain conditions[2], such as traditional enhanced biological phosphorus removal (EBPR) of anaerobic/aerobic (A/O) process, where phosphate accumulating organisms (PAOs) are able to take up volatile fatty acids (VFAs) and convert them to intracellular poly-β-hydroxyalkanoates (PHA) anaerobically, and ATP required for this process is provided by poly-P degradation. Aerobically, PAOs oxidize PHA to gain energy for excess P uptake[3]. Although A/O process is widely applied in real wastewater treatment, the strong dependence on VFAs of wastewater restricts its further application. The studies showed that abounding VFAs in wastewater were necessary to obtain high efficiency of Pi removal of A/O process[4,5]. Cech et al.[6] and Liu et al.[7] also confirmed this viewpoint, and they found that the increase of glucose in influent would deteriorate the efficiency of Pi removal. Therefore, when the VFAs concentrations of wastewater are low, periodic organic matter supplementation and/or chemical "polishing" may be required to attain compliance, as a result, the operational cost increased.

Our recent study on possible alternatives to the conventional EBPR process has demonstrated that it is possible to increase the level of Pi removal using glucose as the sole carbon source under single-stage oxic condition[8−10]. Further research confirmed that Pi removal was achieved by biological process. This phenomenon may enrich the biological Pi removal theory and form a potentially economical and simple strategy for the "one-step" removal of Pi and organic substrates from wastewater. However, the components of real wastewater are complex, and several organic carbon sources such as macromolecular organic compounds, and VFAs exist. To date, whether Pi removal under single-stage oxic condition is only dependent on certain substrate (such as glucose) like A/O process or not is still unknown, which directly decides if single-stage oxic process can be applied in real wastewater treatment. Therefore, the aim of this paper is to evaluate the efficiency of Pi removal under single-stage oxic and traditional A/O conditions using two typical substrates (glucose and acetate), and to investigate the effects of substrates on Pi removal as well as to discuss the probable effecting mechanisms under single-stage oxic condition.

2 Materials and methods

2.1 Experimental device

Experiments were carried out in four reproductive sequencing batch reactors (SBRs) (R1and R2; R1′ and R2′) made of Lucite, with each reactor having a working volume of 12 L (diameter, 12 cm; height, 100 cm).

2.2 Synthetic media

Synthetic wastewater was used in this research. R1, R2 and R1′, R2′ were fed, respectively, with glucose and acetate, but they had almost the same influent amount of carbon element (about 15 mmol/L). The concentrations of other nutrients in the synthetic media fed to the four SBRs are the same as below: PO_4^{3-}-P (35 mg/L), NH_4Cl (40 mg/L), $MgSO_4{\cdot}7H_2O$ (5 mg/L), $CaCl_2$ (5 mg/L), and 0.5 mL of trace metals solution. The trace metals solution has been described in ref. [11].

2.3 Inoculated sludge and operational methods

Inoculated sludge was taken from the First Municipal Wastewater Treatment Plant of Changsha, China, and the initial concentrations of mixed liquor suspended solids (MLSS) in the four reactors were all set at around 4000 mg/L. Since the First Municipal Wastewater Treatment Plant of Changsha operates with an anaerobic-aerobic process, microorganism species are abundant in the inoculated sludge, containing PAOs, nitrobacteria/denitrifying bacteria and other heterotrophic bacteria.

The operation of the four SBRs is described below:

R1and R2: influent→aeration (4 h)→settling/decanting (0.5 h)→idle zone (7.5 h);

R1′ and R2′: influent→anaerobic zone (2 h)→aeration (4 h)→settling/decanting (0.5 h)→idle zone (1.5 h).

For each SBR cycle, 7.8 L supernatant was discharged after the settling period, and the sludge retention time (SRT) in the four SBRs was maintained in approximately 12 days. During the process, a little metal chelating agent and nitrification inhibitor were added to the synthetic media to eliminate the effects of metal phosphorus and nitrification on biological Pi removal. The pH was controlled at 7−8 during aerobic periods through addition of 0.5 M HCl and 0.5 M NaOH.

2.4 Analytical methods

TP was determined by phosphoantimomolybdate ascor-

bic acid spectrophotometry[12], mixed liquor suspended solids (MLSS) and MLVSS were measured by standard method[12], glycogen was measured by the phenol-sulfuric method with glucose as standard[13], total organic carbon (TOC) was determined using a TOC analyzer (ShimadzuTOC-500, Japan), and sludge TP content was measured by the method described in ref. [14].

PHA was measured with gas chromatography (GC)[15,16]. About 50 mL sludge samples were mixed with formaldehyde at a ratio of approximately 1% formaldehyde per sample volume in order to inhibit biomass activity in the sludge. The samples were centrifuged and the supernatant was removed, and then washed with a phosphate buffer solution, and re-centrifuged, and the supernatant was decanted once more. All samples were then lyophilized through a freeze drying unit operated at −54℃ and 0.1 mbar for at least 20 h. Approximately, 20 mg of lyophilized sludge was added to 2 mL chloroform and 2 mL acidified methanol solution, then the samples were digested tightly at 100℃ in an oven for 7 h and cooled to room temperature. Distilled water (2 mL) was then added and mixed vigorously with each sample to remove particulate debris from the chloroform phase and prevent degradation of the GC column. After mixing, setting time of 1 h was allowed to achieve phase separation. The chloroform (bottom) phase was then injected into the GC column. The chromatography was operated with a DB-5 column (30 m length × 0.25 mm LD × 0.25 μm film), a spilt injection ratio of 1∶15 and helium as the carrier gas (1.5 mL/min). A flame ionization detection (FID) unit was operated at 300℃ at an injection port temperature of 250℃. The oven temperature was set at 80℃ for 1 min, increased to 120℃ at 10℃/min , and then to 270℃ at 45℃/min and held for 3 min.

3 Results

3.1 Comparison of multi-parameters in R1 and R2 with those in R1′ and R2′ during steady-state operation

Since March 2008, comparison of performances of Pi removal in R1and R2 with those in R1' and R2' was made, and three repeated experiments were conducted, and the results are shown in Tables 1 and 2.

As shown in Table 1, quite different capabilities of TP removal were shown in R1 and R2. High efficiency of TP removal was obtained in R1, where TP removed was 31.75－33.02 mg/L, TP removed per MLVSS and TP content of MLSS were above 6.7 and 103 mg/g, respectively. The results are similar to the efficiency of TP removal conducted by other researchers using traditional

Table 1 Comparison of multi-parameters in two SBRs with the single-stage oxic process during steady-state operation

	R1			R2		
	Max	Min	Avg	Max	Min	Avg
TP in effluent (mg/L)	3.25	1.98	2.32	26.66	25.13	25.38
TP removed (mg/L)	33.02	31.75	32.68	9.87	8.34	9.62
Efficiency of TP removal (%)	94.3	90.7	93.4	28.2	23.8	27.5
MLSS (mg/L)	5082	4543	4796	3241	2758	2937
MLVSS (mg/L)	3247	2806	3052	2663	2036	2291
MLSS/MLVSS (g/g)	0.65	0.60	0.62	0.80	0.77	0.78
TP removed per MLVSS (mg/g)	7.4	6.7	7.1	3.2	2.7	3.0
TP content of MLSS (mg/g)	118	103	112	65	46	57

Table 2 Comparison of multi-parameters in two SBRs with the traditional anaerobic/aerobic process during steady-state operation

	R1′			R2′		
	Max	Min	Avg	Max	Min	Avg
TP in effluent (mg/L)	32.85	30.13	31.29	20.82	19.58	20.07
TP removed (mg/L)	4.87	2.15	3.71	15.42	14.18	14.93
Efficiency of TP removal (%)	13.9	6.1	10.6	44.1	40.5	42.7
MLSS (mg/L)	4658	4236	4452	4308	3833	4147
MLVSS (mg/L)	3956	3613	3829	3173	2819	3027
MLSS/MLVSS (g/g)	0.89	0.84	0.86	0.75	0.69	0.73
TP removed per MLVSS (mg/g)	0.8	0.4	0.7	3.5	2.9	3.3
TP content of MLSS (mg/g)	27	13	21	93	79	85

A/O process under laboratory conditions[17], and are better than the results obtained in this study using traditional A/O process (Table 2). Compared with R1, R2 had lower efficiency of TP removal, where average TP removed and TP removed per MLVSS were about 9.62 mg/L and 3.0 mg/g, respectively. The capability of TP removal in R2 was better than that in R1′ and was lower than that in R2′ (Table 2). Furthermore, the lower VSS/SS ratio shown in R1 also implied that there was a higher level of poly-P accumulated in the sludge[18]. Besides, Table 1 also shows much higher concentrations of MLVSS and MLSS in R1 than those in R2.

3.2 Transformations of TP, glycogen and PHA during one cycle

Intracellular storage of glycogen and PHA are important energy deposited and supplied materials, and play very important roles in the metabolisms of poly-P organisms[19]. To discuss the reason for the great differences in capabilities of TP removal between R1 and R2, the varieties of TP, glycogen and PHA in two systems during a process of twelve cycles were analyzed, which are shown in Figures 1 and 2.

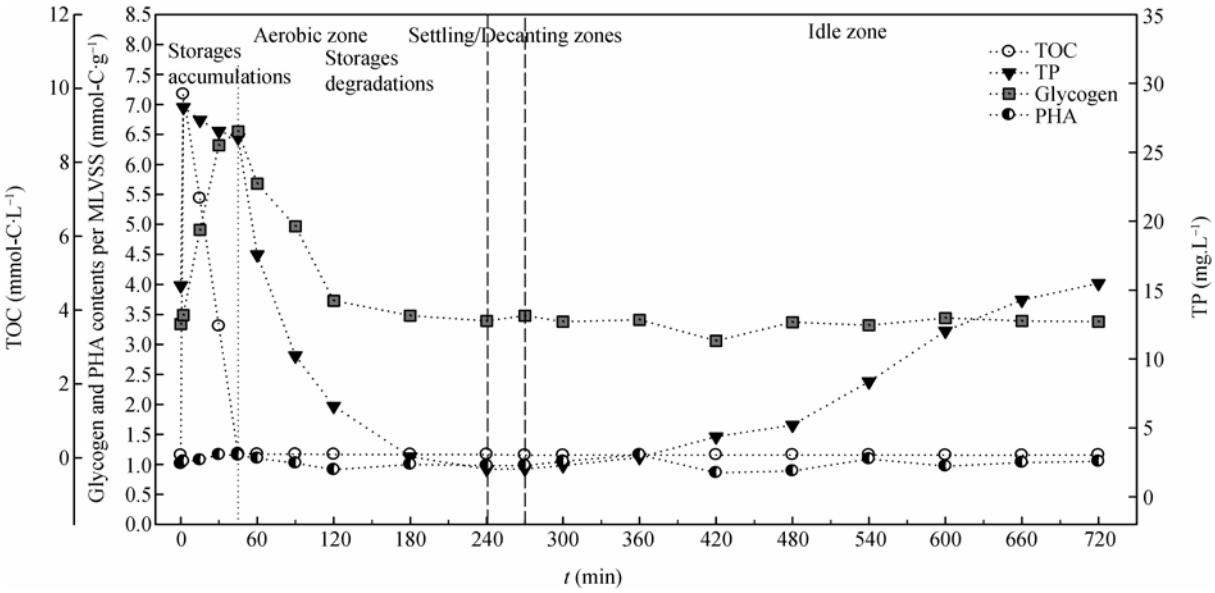

Figure 1 Variations of TP, TOC and sludge glycogen as well as sludge PHAs during one cycle in R1.

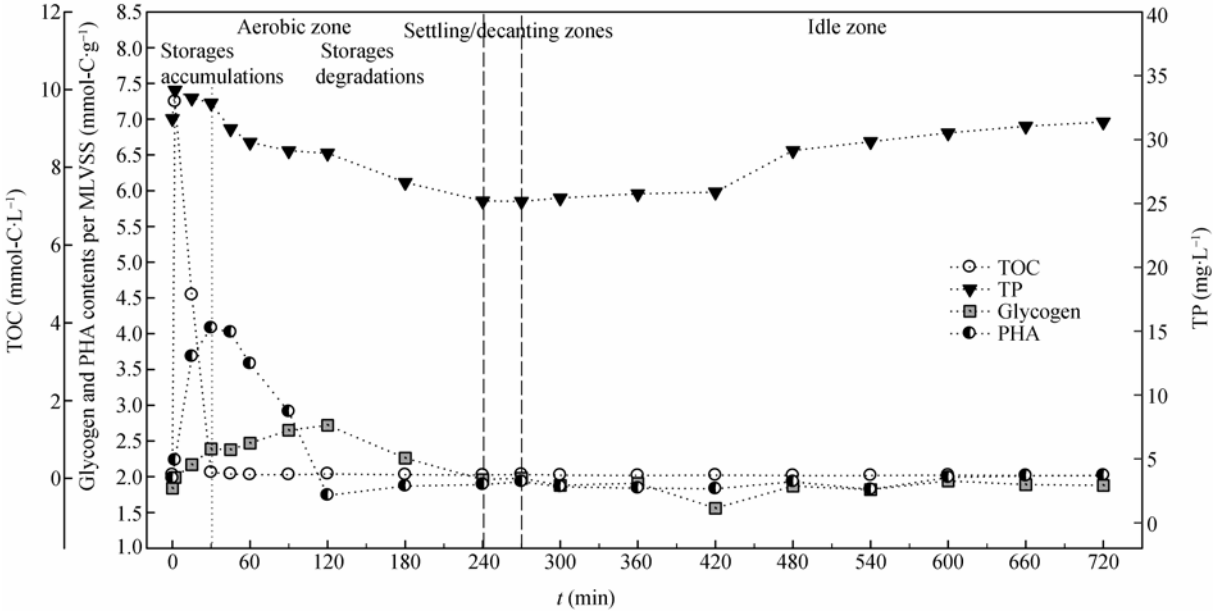

Figure 2 Variations of TP, TOC and sludge glycogen as well as sludge PHAs during one cycle in R2.

As shown in Figures 1 and 2, along with the rapid decreases of external substrates (TOC) at the beginning of aeration, significant internal storage compounds are accumulated in both SBRs, but the species and the contents of internal storage compounds are obviously different. Dominant internal storage of glycogen was accumulated in R1 (max accumulation of 3.21 mmol-C/g), and the similar phenomenon was observed by Dirck et al.[20] and Carucci et al.[21] using activated sludge process. Although obvious accumulations of PHA and glycogen were observed in R2, the main storage polymer was PHA (2.1 mmol-C/g of PHA vs 0.55 mmol-C/g of glycogen), moreover, the total contents of storages (2.65 mmol-C/g) were also lower than those in R1 (3.21 mmol-C/g). However, just a slight TP removal was observed in both SBRs during the period of storage accumulation (2.17 and 1.01 mg of TP/L in R1 and R2, respectively). When external substrates were depleted, the contents of glycogen decreased in R1, and nearly decreased to the initial level after aeration; PHA also decreased to the initial level after 2 h of aeration in R2, but glycogen was continuing to accumulate to a max content of 0.88 mmol-C/g during the period of PHA degradation, and then gradually decreased to the initial level. During the transformations of internal storage compounds, obvious TP removal was observed in both SBRs, and the concentrations of TP in R1 and R2 were 2.03 and 25.15 mg/L in effluent, respectively. During the idle zone, inconspicuous changes of glycogen and PHA were observed other than a high level of Pi release in both SBRs, and the microorganisms in R1 had a higher Pi release than those in R2, which indicated that poly-P was hydrolyzed in two SBRs, and the contents of poly-P hydrolysed in R1 were higher than those in R2 too.

4 Discussion

Preliminary studies showed that the fundamental reason for excess Pi uptake is the setting of idle starvation period in SBR with single-stage oxic process, and energy required for cell maintenance is provided by poly-P hydrolysis, so that poly-P plays an important role in microbial metabolism, inducing excess Pi uptake to recover intracellular poly-P level. From Figures 1 and 2, it can be seen that long idle starvation periods existed in both R1 and R2, and carbon contents and other operating conditions were identical except for substrates. Although excess Pi uptake was clearly observed in the two systems, why there were so large differences in Pi removal performances between R1 and R2 remains unclear.

Although the status of poly-P playing in the microbial metabolism decides its ability of excess synthesis, poly-P is a storage material stored *in vivo* in micro-organisms, and a certain consumption of ATP is required for its synthesis. Maurer et al. considered that synthesis of 1 mol each poly-P would consume 1.2 mol of ATP[22], which indicated that poly-P synthesis during aeration was also subjected to the available ATP. In this study, TOC was quickly consumed at the beginning of aeration, and simultaneously, substantial internal storage was accumulated *in vivo* micro-organisms. Thus aerobic period of this study could be divided into two parts: external substrates consumption period (energy storage material accumulation period) and internal storage consumption period (energy storage material oxidation/ conversion period), as shown in Figures 1 and 2. Inconspicuous TP decreases were observed in the external substrates consumption period of both systems, which indicated that ATP produced by the external substrates oxidization was mainly supplied for intracellular storage accumulation rather than poly-P synthesis; in the internal storages consumption period, most of the TP removals in two systems were realized during this period, which indicated that ATP required for poly-P synthesis in this study was provided by the oxidation of internal storage. Biochemical studies showed that the microbial internal carbon source is oxidized into CO_2 and water through the citric acid cycle (TCA cycle) and oxidative phosphorylation and generates substantial ATP during aeration. Furthermore, ATP generated from the same carbon content of different carbon sources (such as quality of glycogen, protein and PHA) is almost equal[23,24]. Because more internal storage was accumulated by per MLVSS in R1 during the external substrates consumption than that in R2 (R1: 3.21 mmol-C /g; R2: 2.65 mmol-C /g (Figures 1 and 2)), the internal storage accumulated in both systems was almost oxidized. Except the energy required for cell growth and maintenance, more ATP was provided for poly-P synthesis in R1, demonstrating better capability of Pi uptake in R1.

In addition, different types of substrates were fed to R1 and R2, and though they had almost the same influent amount of carbon element, the accumulations of internal storages were obviously different in their respec-

tive system, therefore, researchers explored the reason why per MLVSS has different accumulations of internal storage during the period of consumptions of external substrates in two systems.

During the process of aerobic respiration, the oxidation of glucose is divided into two stages[23,24]: (1) glycolysis, where glucose is reduced to an intermediate product-pyruvate via the EMP pathway; (2) aerobic decomposition of pyruvic acid, i.e., TCA cycle (TCA). At the stage of glycolysis, glucose is transformed into pyruvate by the path of phosphorylation, aldolase catalysis, substrate-level phosphorylation etc. This process does not require oxygen consumption, and has only a small amount of energy release (oxidation of 1 mol glucose net 2 mol ATP); at the stage of aerobic decomposition, pyruvate is transformed into acetyl-CoA, and then acetyl-CoA enters the TCA cycle and finally is oxidized to CO_2 and water completely, which will produce a lot of ATP (1 mol pyruvate generates 15 mol ATP by the TCA cycle). Since glucose oxidation generates a lot of ATP, there is a certain amount of ATP remaining apart from ATP used in cell growth. However, ATP could not exist in a certain form for a long period of time for ATP is a free energy storage element (i.e., ATP is not the energy storage form, but a molecular energy transfer)[24], thus the remaining ATP can supply high-energy phosphate bond through glucose phosphate (glucose-6-phosphate, and glucose-1-phosphate) to form the final energy storage of glycogen (Figure 3), which is the phenomenon of glycogen accumulation.

During the aerobic respiration of sodium acetate, in addition to TCA cycle, bacteria can also use sodium acetate to precede glyoxylate pathway (Figure 4). Sodium acetate can transform into acetyl-CoA by acetylating, and then acetyl-CoA may simultaneously enter TCA cycle and glyoxylate pathway, synthesizing energy storage of PHA using the residual ATP generated by TCA cycle (1 mol $NADH_2$ electron transport system through the oxidation of NAD can generate 3 mol ATP). During glyoxylate pathway, isocitrate decomposes into glyoxylate and succinate, and glyoxylate forms malate after acetylation enters TCA cycle. Additionally, succinate can be transported to mitochondria other than entering TCA cycle, where succinate transforms to oxaloacetate and synthesizes storage of glycogen via gluconeogenesis pathway (Figure 4), which can also explain why there exists a accumulation of a small amount of glycogen during external substrate consumption period in R2.

Comparing the accumulation ways of internal storage of R1 with those of R2, we could see that no extra ATP was consumed during the glycogen accumulation process but a small amount of ATP was consumed in the process where glucose-6-phosphate was generated from glucose via phosphorylation. However, during the period of internal storages accumulations in R2, PHA accumu-

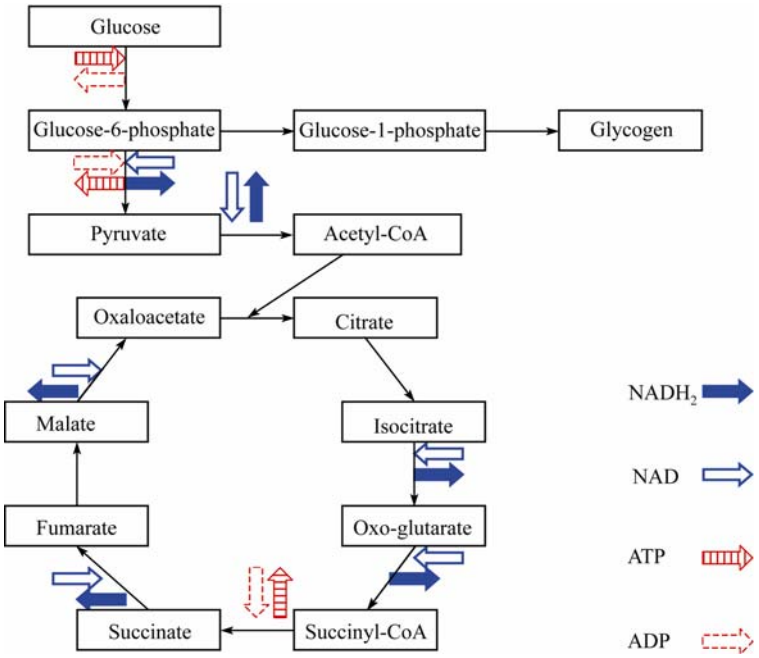

Figure 3 Aerobic metabolism of glucose.

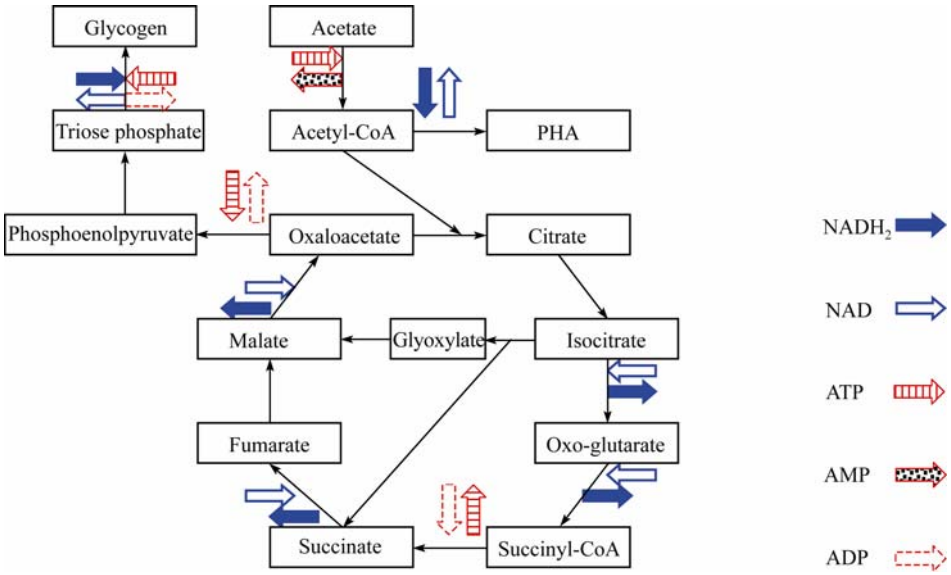

Figure 4 Aerobic metabolism of acetate.

lation from acetyl-CoA would consume $NADH_2$ (1 mol $NADH_2$ produces 3 mol ATP under aerobic conditions) and acetyl-CoA formation would consume ATP. Meanwhile, the synthesis of glycogen from oxaloacetate via the gluconeogenesis pathway would also consume ATP and $NADH_2$. These indicated that more ATP was consumed during the aerobic metabolism of sodium acetate in R2. Since the two systems had the same influent amount of carbon element and equivalent carbon contents of external substrate decomposed by TCA cycle generating almost equal ATP[24], bacteria in R2 needed to oxidize more acetate to produce more ATP to synthesize the same amount of internal storage as that in R1, resulting in lower contents of internal storage in R2. In addition, the facts also explained why R2 had lower MLVSS concentrations than that in R1 (Table 1).

As elucidated in the foregoing, this study confirmed that biological Pi removal could be achieved in SBR with single-stage oxic process using different substrates and discussed the probable mechanism. Only 27.5% of average efficiency of Pi removal was obtained in R2, but it was caused by increasing the Pi concentration of influent to investigate the max amount of Pi uptake, and besides Pi uptake for cell normal assimilation, excess Pi uptake was observed in R2 too. The researches showed that TP content in sludge accounted for 1%—2% of cell dry weight, but sludge TP content in R2 arrived at 46—65 mg/g, which was similar to the performance of Pi removal conducted in pilot-scale by Mullan et al.[2]. At present, Pi concentration in influent of municipal wastewater was about 10 or 5—7.5 mg/L[19], and the average amount of Pi removal 9.62 $mg \cdot L^{-1}$ showed that effective Pi removal could be obtained from wastewater even using acetate as the sole carbon source. Therefore, the process had obvious advantage for the municipal wastewater because of its low Pi concentration but massive quantity of wastewater treated daily. Now, we are going to investigate the effects of other substrates on the efficiency of Pi removal and the microbiological communities to establish the biological Pi removal theory under aerobic condition, and make the process become applied in real wastewater treatment successfully.

5 Conclusions

Biological Pi removal could be obtained in SBR with single-stage oxic process using glucose (R1) and acetate (R2) as the sole carbon sources, but the carbon type had a great effect on Pi removal. After steady operation, the amounts of Pi uptake were 6.7—7.4, and 2.7—3.2 mg/g, respectively. The main accumulation of internal storage was glycogen; on the contrary, PHA was the main storage while the level of glycogen accumulation was low during the period of external substrate consumption. Moreover, the contents of internal storage were higher in R1 than those in R2. The results suggested that different aerobic metabolism of substrate occurred in R1 and R2, resulting in aerobic storage accumulated in bacteria of R1 and R2. As a result, ATP content provided for Pi uptake was different in R1 and R2, and the capability of Pi uptake was also different from each other.

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